

The effect of certolizumab drug concentration and anti-drug antibodies on TNF neutralisation

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Abstract

Objective

Tumour necrosis factor (TNF) inhibitors like certolizumab, elicit an immunogenic response leading to the formation of anti-drug antibodies (ADAs). We sought to mechanistically investigate the relationship between certolizumab concentrations, ADAs, and the effective TNF neutralising capacity in sera of rheumatoid arthritis (RA) patients. TNF neutralising capacity of certolizumab was compared to the neutralising capacity of adalimumab.

Methods

Serum samples were collected from 40 consecutive certolizumab-treated RA patients at baseline and 4, 16, 28 and 52 weeks after treatment initiation [Dutch Trial Register NTR (Nederlands Trial Register) Trial NL2824 no. 2965]. Certolizumab concentration and ADA titre were measured with a certolizumab bridging enzyme-linked immunosorbent assay (ELISA) and a drug-tolerant radioimmunoassay (RIA), respectively. TNF neutralisation by certolizumab and adalimumab, in presence or absence of ADAs, was analysed with the TNF-sensitive WEHI bioassay.

Results

Despite a high incidence of ADAs during one year of follow-up (65%; 26/40 patients), certolizumab levels of >10 µg/ml were measured in most patients. The capacity for TNF neutralisation highly correlated with certolizumab serum concentration, whereas no association with ADAs was observed. Similar results were obtained for adalimumab. The relative in vitro neutralising potency was higher for certolizumab compared to adalimumab.

Conclusion

Anti-certolizumab antibodies were detected in a large proportion of patients, but in most cases where ADAs were detected, certolizumab was also present in high concentrations, directly correlating with in vitro neutralising capacity. These results indicate that measurement of certolizumab drug levels, rather than ADAs, have direct clinical significance.

Key words

certolizumab, anti-drug antibodies, TNF, rheumatoid arthritis, adalimumab

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Introduction

Biological disease-modifying anti-rheumatic drugs (bDMARDs) have been developed to inhibit the activity of inflammatory cytokines such as tumour necrosis factor (TNF). These TNF inhibitors have proven to be a successful treatment option for patients with rheumatoid arthritis (RA) and other inflammatory disorders (1, 2). Therapeutic antibodies such as certolizumab have been shown to elicit an immunogenic response leading to the formation of anti-drug antibodies (ADAs). However, the reported incidence, and levels of anti-certolizumab antibodies varies highly between different studies (~5–37% of the patients) (2–4). In a recent study by Jani *et al.* detection of ADAs in certolizumab-treated RA patients was not associated with the 12 months European League Against Rheumatism (EULAR) response (4). In contrast, in certolizumab-treated Crohn's disease patients persistent ADAs were correlated with reduced efficacy, while transient ADAs were not (5). These mixed results are seemingly in contrast with many studies demonstrating clear correlations between ADA formation to adalimumab or infliximab and a lower likelihood of minimal disease activity or clinical remission (6–10).

The detection of ADAs in patients varies widely between studies, depending on duration of follow-up, concomitant medication, the type of TNF inhibitor and the type of assay that is used to detect ADAs; a golden standard for the quantification of ADAs is missing (11). In particular, drug concentration may profoundly affect the detection of ADAs, depending on how drug-tolerant the ADA assay is. Importantly, ADAs and drug levels will affect each other mutually.

ADAs are generally expected to only affect treatment efficacy by lowering exposure to free active drug, via neutralisation and/or enhanced clearance. Hence, ADAs might only influence clinical response when they affect pharmacokinetics (PK) to a noticeable degree (12). When enough free active drug is left to bind to its target, despite the presence of ADAs, ADAs are unlikely to impair clinical response. Pre-

viously, we have shown that the antibody response to a range of therapeutic antibodies, including certolizumab and adalimumab, is highly restricted to the antigen binding site, thereby predominantly neutralising (13, 14). In a number of studies, increasing serum concentrations of TNF inhibitors, including certolizumab, were associated with better clinical outcome (4, 7, 15–18). Furthermore, the amount of TNF inhibition will depend on the strength of binding between TNF inhibitor and ADAs on the one hand and TNF inhibitor and TNF on the other hand. In other words, the balance between TNF inhibitor concentration, ADA titre and TNF concentration plays a role in determining the TNF neutralising efficacy of the TNF inhibitor.

In the present study we describe the incidence of anti-certolizumab antibodies, as well as the relationship between serum certolizumab concentrations and the TNF neutralising capacity in presence and absence of ADAs. We compared the certolizumab neutralising capacity with the neutralising capacity of adalimumab, since these drugs are structurally different and have a different binding strength for TNF (19).

Materials and methods

Details about the methodology can be found in the Supplementary file. Briefly, certolizumab concentration and anti-certolizumab antibody titre were measured with a rabbit anti-certolizumab bridging ELISA and a one-tiered or two-tiered certolizumab RIA, respectively, in 40 consecutive RA patients starting certolizumab treatment [Dutch Trial Register NTR (Nederlands Trial Register) Trial NL2824 no. 2965]. The study was approved by the medical ethics committee of the Slotervaart Hospital and Reade Medical Research Ethics Committee, Amsterdam, the Netherlands (CCMO NL35209.048.11). All patients gave written informed consent. To determine the TNF neutralising activity of certolizumab in patient sera, in presence or absence of ADAs, the TNF-sensitive WEHI bioassay was used. The TNF neutralising capacity of certolizumab was compared with the neutralising capacity of adalimumab. A

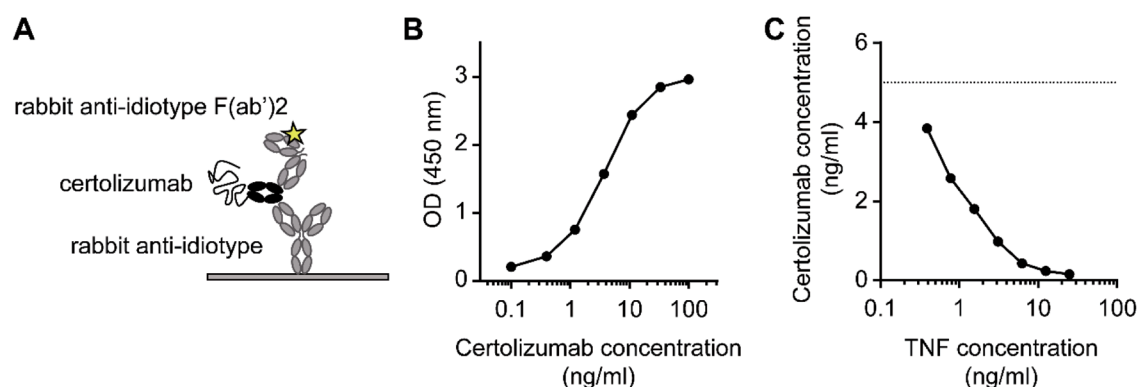


Fig. 1. Development of bridging ELISA for certolizumab. A cross-linking assay in which for both capture and detection polyclonal rabbit anti-idiotype antibodies were used, resulted in a sensitive assay to quantify certolizumab serum concentrations.

A: Schematic overview of the certolizumab concentration assay. Certolizumab in serum is captured by polyclonal rabbit anti-certolizumab antibodies. Subsequently, biotinylated F(ab')₂ fragments of the same polyclonal rabbit anti-certolizumab antibodies are added for detection of certolizumab.

B: Representative calibration curve of the certolizumab concentration assay.

C: Inhibition of certolizumab specific signal by increasing concentration of TNF. The concentration assay does not detect certolizumab bound to its target, assuring that only functional certolizumab is measured in this assay.

Table I. Demographics, previous and concomitant therapies, and disease status at baseline

	Patients (n=40)
Demographics	
Age, median (IQR) (years)	56 (47-63)
Female, no. (%)	30 (75)
BMI, median (IQR)	24.4 (22.0-27.8)
DMARD therapy	
Previous Biologic, no. (%)	20 (50)
Previous DMARDs, median (IQR)	1 (0-2)
DMARDs use, no. (%)	34 (85)
MTX use, no. (%)	28 (70)
MTX dose, median (IQR) (mg/week)	22.5 (15-25)
Prednisone use, no. (%)	11 (28)
Prednisone dose, median (IQR) (mg/day)	5 (5-10)
Disease Status	
Disease duration, median (IQR) (years)	8 (3-16)
ACPA positive, no. (%)	30 (75)
IgM-RF positive, no. (%)	23 (58)
Erosive, no. (%)	16 (40)
DAS28, median (IQR)	4.6 (3.2-5.3)
ESR, median (IQR) (mm/hour)	20 (9-37)
CRP, median (IQR) (mg/litre)	6 (2-17)
Tender joint count, median (IQR)	6 (4-9)
Swollen joint count, median (IQR)	3 (2-6)

IQR: inter quartile range; no: number; BMI: body mass index; DMARD: disease-modifying anti-rheumatic drug; MTX: methotrexate; ACPA: anti-citrullinated protein antibody; IgM-RF: immunoglobulin M rheumatoid factor; DAS28: Disease Activity Score in 28-joints; ESR: erythrocyte sedimentation rate; CRP: C-reactive protein.

selection of samples was made, to represent a range of serum certolizumab (4.8–60 µg/ml) or adalimumab concentrations (0.52–32.5 µg/ml) and ADA ti-

tres (20–830 AU/ml anti-certolizumab antibodies and 30–113 AU/ml or 30–1380 AU/ml anti-adalimumab antibodies, detected with the drug-sensitive RIA or drug-tolerant ARIA, respectively). This selection was irrespective of any other (clinical) parameter.

Results

Development of a rabbit anti-certolizumab bridging ELISA

In order to measure certolizumab levels in certolizumab-treated RA patients, we designed a bridging ELISA using polyclonal rabbit anti-idiotype antibodies for both capture and detection (Fig. 1A). This approach resulted in a highly sensitive assay with a quantifiable range between 0.1 and 120 µg/ml (Fig. 1B). The same principle was previously demonstrated to be valid by using rabbit anti-natalizumab antibodies and natalizumab Fab (20).

An advantage of this certolizumab bridging ELISA format is that it allows specific detection of certolizumab. To test whether this assay only detects functional certolizumab, we conducted an inhibition experiment with TNF. Almost complete inhibition of assay response was observed when excess TNF was titrated into a sample containing 5 ng/ml certolizumab (Fig. 1C). In other words, if the TNF binding site of certolizumab is blocked with TNF it is no longer detected in this assay. Therefore, we anticipate that if the TNF binding site would be blocked by anti-idiotype

antibodies formed in a patient, this fraction - that will no longer be active - will also not be measured. This assures that only functional, free certolizumab is measured in this assay.

Patients

Over one year, 147 serum samples from 40 consecutive RA patients who started treatment with certolizumab were included. The median age was 56 years and 75% was female. The majority of patients was concomitantly treated with MTX (70%) and half of the patients was previously treated with a different biologic. Patients' baseline characteristics are shown in Table I. 18 patients completed the study follow-up of one year. Twenty-two patients dropped out of the study: twelve due to treatment failure, three due to treatment failure and side effects, five due to adverse events (including recurrent respiratory infections, allergic reactions and psoriasis pustulosis) and two due to withdrawal of their informed consent. This high drop-out rate could probably be explained by the fact that approximately half of the patients were not TNF inhibitor naïve, and thus previously failed treatment with other TNF inhibitors. We observed 6 cases with a drug hypersensitivity reaction. However, there was no significant difference between patients with or without detectable ADAs (respectively 2/26 [7.7%] and 4/14 [28.6%], $p=0.214$).

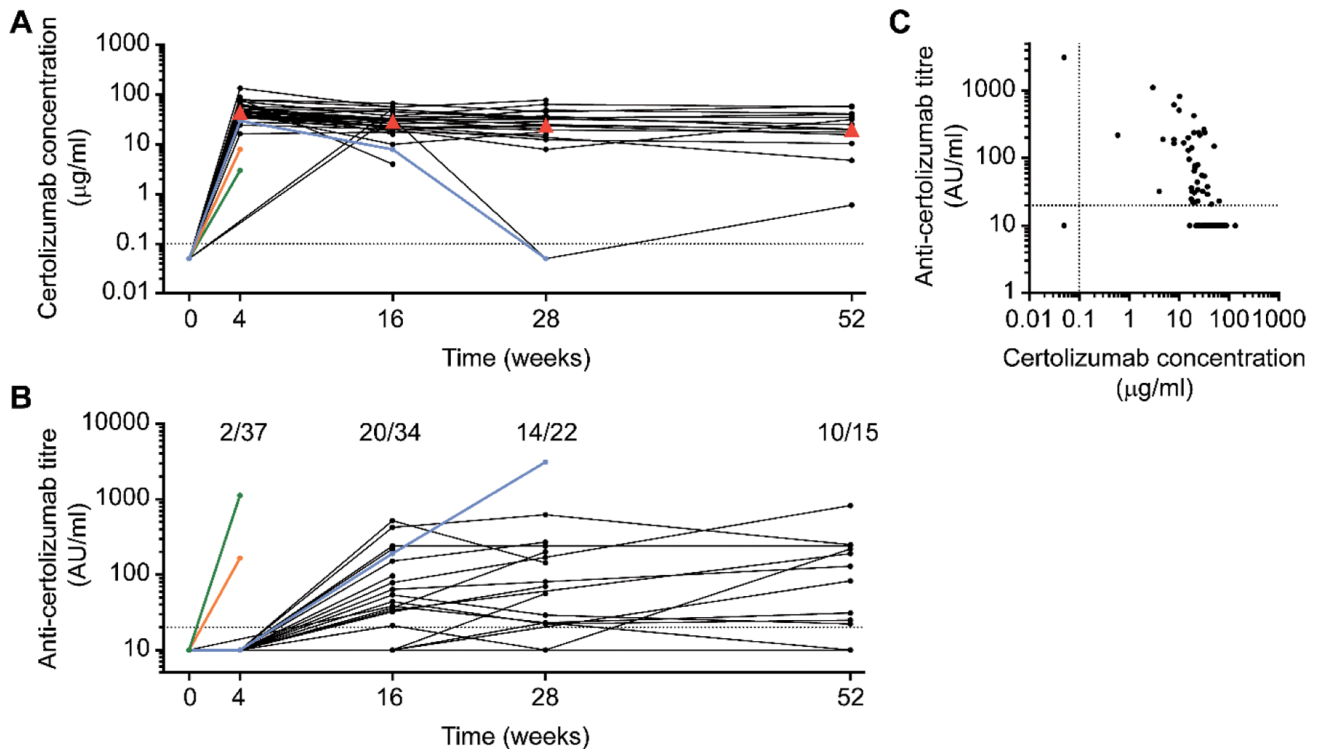


Fig. 2. Longitudinal certolizumab and anti-certolizumab antibody concentration.

A: Longitudinal certolizumab trough serum concentrations (µg/ml) were measured in 40 consecutive certolizumab-treated RA patients during one year follow-up. Red triangles indicate median certolizumab concentration of all patients at the individual time points.

B: Longitudinal anti-certolizumab antibody titres (AU/ml) measured with a drug-tolerant one-step RIA. Numbers above the graph indicate anti-certolizumab antibody positive samples / total sample number.

A-B: Solid lines connect measurements at different time points within a patient. Certolizumab concentrations and anti-certolizumab antibodies in orange, green and blue are from the same individual, respectively.

C: Correlation between certolizumab concentration and anti-certolizumab antibody titre (Spearman's $\rho = -0.688$, $p < 0.0001$ ($n = 108$)). Each dot represents the combined certolizumab trough level and ADA titre from a single individual at one time point. Baseline samples were excluded.

A-C: Dashed lines indicate LLOQ of 0.1 µg/ml certolizumab or LOD of 20 AU/ml anti-certolizumab antibody.

Longitudinal certolizumab and anti-certolizumab antibody concentrations

An initial loading dose resulted in certolizumab trough levels reaching maximum concentration at week four after treatment initiation. Substantial variation in certolizumab trough levels was observed between patients, with concentrations of 45.0 (3.00–136) µg/ml, 29.5 (4.00–67.0) µg/ml, 24.5 (0.1–78.0) µg/ml and 20.4 (0.6–59) µg/ml (median (inter quartile range (IQR))) at week 4, 16, 28 and 52, respectively. Despite inter-patient variation in certolizumab concentration, longitudinal intra-patient variation was minimal; in 58% of the patients certolizumab concentrations remained stable in time within a factor three (Fig. 2A).

Next, we investigated anti-certolizumab antibody formation with the drug-tolerant one-step RIA (21). Although in two patients (5%) ADAs could already be detected after four weeks of treat-

ment (green and orange line, Fig. 2B), in most patients with detectable anti-body formation, ADAs could be detected from week 16 onwards. Overall, the incidence of ADA positivity during one year of follow-up was 65% (26/40 patients). ADA titres of the ADA positive patients were 166 and 1120 AU/ml ($n = 2$) at week four and 44 (21–517) AU/ml, 112 (22–3120) AU/ml and 160 (22–830) AU/ml (median (IQR)), at week 16, 28 and 52, respectively. Of all patients who completed the one-year follow-up, two patients had an apparent transient ADA response.

The two patients with detectable ADAs at week four also had the lowest certolizumab concentrations at that point (green and orange line, Fig. 2A and B). Moreover, the patient with the highest antibody concentrations overall had undetectable certolizumab at week 28 and discontinued treatment at that time, due to skin abnormalities (blue line, Fig.

2A and B). Another patient had undetectable certolizumab at week 28, but ADAs could only be detected at week 52. In the majority of patients, levels of >10 µg/ml certolizumab were detected despite the simultaneous detection of ADAs (Spearman's $\rho = -0.688$, $p < 0.0001$; Fig. 2C). Therefore, we hypothesised that in the majority of ADA-positive patients, certolizumab can still exert its TNF neutralising function.

TNF neutralisation by certolizumab correlates with drug concentration

To further examine the relationship between anti-TNF, ADAs, and neutralisation capacity, we tested TNF neutralising activity of patient sera with a TNF-sensitive WEHI bioassay. This assay quantifies killing of WEHI cells by biologically active TNF (Fig. 3A). Killing of WEHI cells can be rescued by neutralisation of TNF by certolizumab or adalimumab in serum. However,

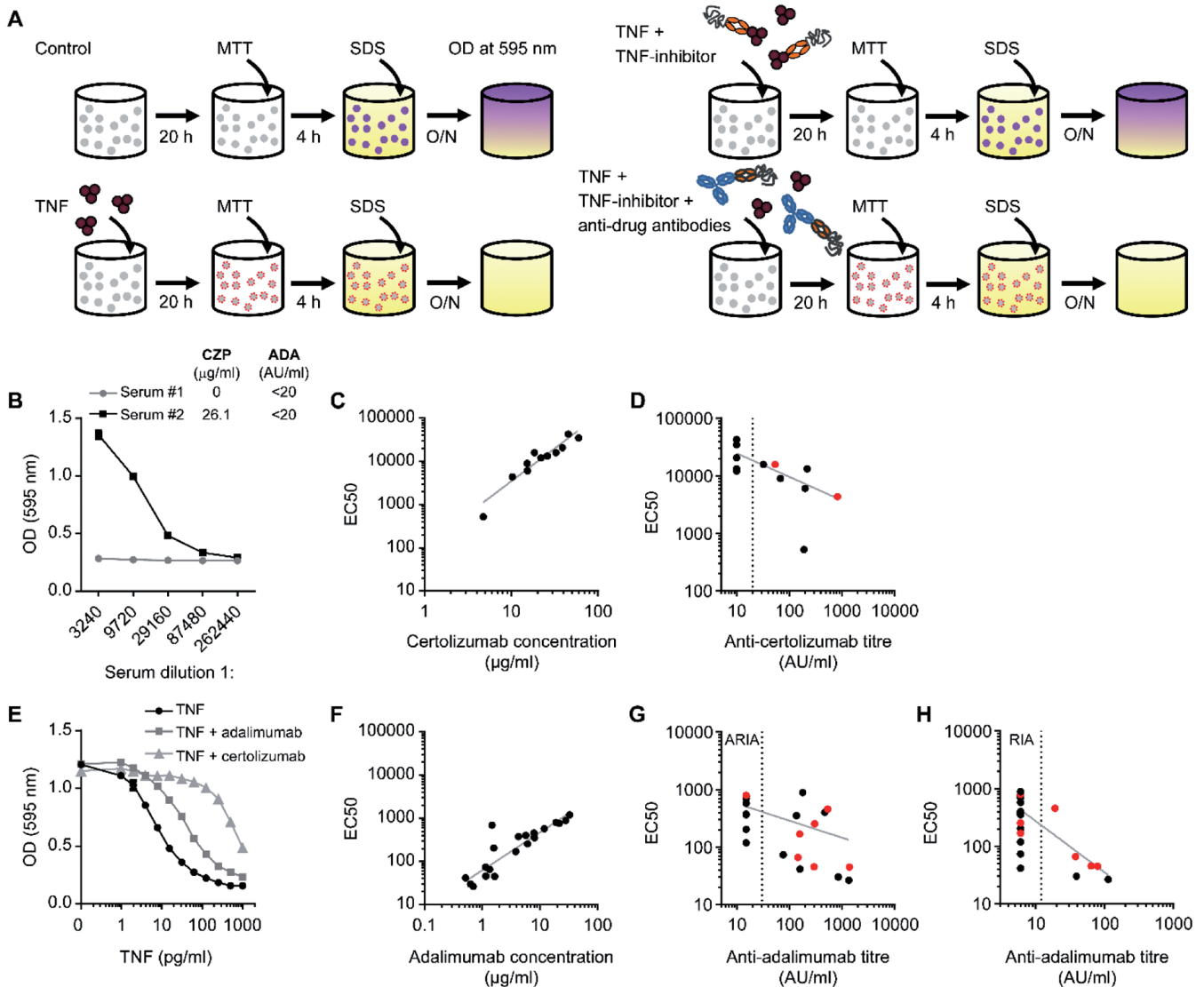


Fig. 3. TNF neutralisation by certolizumab and adalimumab serum samples, in presence or absence of ADAs.

A: Schematic representation of the TNF-sensitive WEHI bioassay, used to analyse TNF neutralising activity. The intensity of the purple color, measured at 595 nm in a microtitre plate reader, is used as a readout for cell viability. TNF neutralisation by certolizumab or adalimumab in serum samples rescues WEHI cells (right upper panel), whereas presence of ADAs abolishes this protective effect (right lower panel).

B: Serum samples were serially diluted and incubated with 100 pg/ml TNF to determine TNF neutralisation. Decreasing drug concentration resulted in reduced TNF neutralisation, as represented by decreasing OD. A representative certolizumab serum sample is shown (Serum #2), and a baseline certolizumab sample was included as negative control (Serum #1). Data expressed as mean \pm SD of duplicate measurements.

C-D: Correlation between TNF neutralisation, as expressed by EC50 values, and (C) certolizumab concentration (Pearson $r=0.909$, $p<0.0001$ ($n=12$)) or (D) anti-certolizumab antibody titre (Pearson $r=-0.471$, $p=0.122$ ($n=12$)).

E: Bioactivity of a titration of TNF incubated in presence or absence of 5 ng/ml certolizumab or adalimumab. Data expressed as mean \pm SD of duplicate measurements. Graph depicts representative data of three independent experiments.

F-H: Correlation between TNF neutralisation, as expressed by EC50 values, and (F) adalimumab concentration (Pearson $r=0.900$, $p<0.0001$ ($n=21$)) or (G) anti-adalimumab antibody titre, measured with the drug-tolerant ARIA (Pearson $r=-0.434$, $p=0.049$ ($n=21$)) (H) or measured with the drug-sensitive RIA (Pearson $r=-0.500$, $p=0.021$ ($n=21$)).

D, G-H: Red dots represent samples derived from patients that did not use methotrexate, black dots represent samples derived from patients concomitantly treated with methotrexate. Stratification by methotrexate did not change the results.

C-D, F-H: Grey lines indicate log-log linear fit, weight by $1/Y^2$.

the presence of ADAs can abolish this protective effect. A selection of patient samples was made (see *Materials and methods*), to represent a range of serum certolizumab concentrations and ADA titres, irrespective of any other (clinical) parameter. These samples were

serially diluted and 100 pg/ml TNF was added to determine the neutralising capacity. Subsequently, these samples were added to the WEHI cells and after 24 hours TNF neutralisation was determined with the MTT-reduction method. The EC50 of all samples was

determined (as described in *Materials and methods*), with higher EC50 values representing larger TNF neutralising capacity.

Healthy donor serum and baseline patient samples were tested as negative controls and were indeed not able to

neutralise TNF ($EC_{50} < 40$; serum #1 in Fig. 3B). TNF could be dose-dependently neutralised by certolizumab, as shown by a representative certolizumab serum sample titration in Figure 3B (serum #2). Next, we analysed TNF neutralising activity in the above-mentioned selected serum samples, with different certolizumab concentrations and ADA titres. The TNF neutralising activity is expressed by an EC_{50} value. TNF neutralisation was highly correlated with certolizumab serum concentration (Pearson $r = 0.909$, $p < 0.0001$; Fig. 3C). By contrast, no correlation was observed between anti-certolizumab antibodies and TNF neutralisation (Pearson $r = -0.471$, $p = 0.122$; Fig. 3D). Despite the presence of ADAs, certolizumab could still exert its TNF neutralising activity.

Since certolizumab is a Fab domain and thus structurally different from IgG1 antibodies, we compared TNF neutralisation by certolizumab to the TNF neutralising activity of adalimumab. To investigate whether there is any difference in TNF neutralisation by certolizumab and adalimumab itself, we first analysed TNF bioactivity in the presence or absence of 5 ng/ml certolizumab or adalimumab (Fig. 3E). Although both drugs neutralised TNF, the relative neutralising potency of certolizumab was higher compared to adalimumab. Next, we analysed TNF neutralisation by serum samples from adalimumab-treated RA patients (6). Like certolizumab, adalimumab serum levels were highly correlated with TNF neutralising activity (Pearson $r = 0.900$, $p < 0.0001$; Fig. 3F). A weak correlation was observed between the anti-adalimumab antibody titre, measured with a drug-tolerant ARIA, and TNF neutralisation (Pearson $r = -0.434$, $p = 0.049$; Fig. 3G). Anti-adalimumab antibodies measured with a drug-sensitive RIA assay showed a slightly stronger correlation with TNF neutralisation (Pearson $r = -0.500$, $p = 0.021$; Fig. 3H). However, this correlation was still weak compared to the correlation between adalimumab serum levels and TNF neutralisation. Together, we showed that TNF neutralisation by certolizumab relates directly to drug concentration, but

not anti-certolizumab antibodies. The same applied for adalimumab.

Discussion

The present study describes the relationship between certolizumab concentrations and TNF neutralising capacity, in presence and absence of anti-certolizumab antibodies, using sera derived from certolizumab-treated RA patients. We showed that certolizumab trough concentrations were highest four weeks after initiation of treatment, and in 58% of the patients certolizumab concentrations remained stable in time within a factor three. The minor drop in certolizumab concentration after week four was not associated with the formation of anti-certolizumab antibodies as shown in this study, but could rather be explained by the initial loading dose of certolizumab.

We demonstrated an inverse correlation between anti-certolizumab antibodies and drug concentrations (Fig. 2C). Only in a minority of patients (35%) no anti-certolizumab antibodies could be detected during one year of follow-up. However, in most samples in which anti-certolizumab antibodies were detected, certolizumab serum concentrations remained well above 10 µg/ml, which is within the therapeutic range according to the certolizumab concentration-effect curve (17), irrespective of the presence of detectable ADAs.

We demonstrated that both certolizumab and adalimumab neutralised TNF, although the relative *in vitro* neutralising potency was higher for certolizumab compared to the neutralising potency of adalimumab. Certolizumab binds TNF with higher affinity compared to adalimumab, and can therefore more efficiently bind and neutralise TNF (19). Of note, certolizumab is a monovalent Fab fragment, whereas adalimumab is a bivalent antibody. However, when the concentrations would have been corrected for equal amounts of binding sites, the difference in neutralising efficacy would have been even more pronounced.

For both certolizumab and adalimumab, there was a strong correlation between drug levels and TNF neutralising capacity as measured in the WEHI

bioassay, although we observed higher EC_{50} values for certolizumab than for adalimumab. However, the direct comparison of EC_{50} values should be done with caution, since certolizumab serum concentrations are much higher compared to adalimumab concentrations. The high dosing regimen of certolizumab is remarkable, as we demonstrated efficient TNF neutralisation by certolizumab. Golimumab for example, binds TNF with higher affinity compared to adalimumab (19), and has a lower dosing regimen compared to adalimumab. In striking contrast, certolizumab binds TNF with even higher affinity compared to golimumab, while the dosing regimen of certolizumab is the highest. Nonetheless, dose-finding studies demonstrated a dose-dependent improvement in clinical response with increasing certolizumab doses (22–24). Recently, it was shown that higher certolizumab plasma levels were associated with larger DAS28 improvement from baseline (4, 18), although a plateau effect in Δ DAS with 20 µg/ml certolizumab has been described (17). Since certolizumab efficiently neutralises TNF, it is noteworthy that high certolizumab doses are required for clinical response. There appears to be a discrepancy between *in vitro* efficacy of TNF neutralisation and *in vivo* clinically effective certolizumab concentrations. One might wonder whether local certolizumab concentrations, at the site of inflammation, are lower compared to systemic concentrations. However, as certolizumab effectively entered the inflamed tissue (25, 26), a lack of distribution to the local site of inflammation does not explain this controversy. Furthermore, differences in Fc-Rn-mediated recycling and Fc-mediated clearance between certolizumab, adalimumab and golimumab might contribute to the difference in dosing regimen, given that certolizumab is a Fab domain while adalimumab and golimumab are IgG1 antibodies. These structural differences might also result in different Fc-mediated effector functions, including apoptosis and complement activation (27–29). Finally, it is possible that high certolizumab dosing contributes to the induction of tolerance, thereby suppressing

immunogenicity. This would suggest that after the initial certolizumab loading dose, lower doses of certolizumab might be sufficient to maintain clinical effectiveness. However, the main effect of antibody formation is expected to be lowering the effective drug exposure. In a concentration-effect curve the impact of immunogenicity on effective drug levels c.q. drug exposure is taken into account, and this analysis still suggests added benefit of certolizumab concentrations up to almost 20 µg/ml for RA (17).

In this study, antibodies were detected in 65% of the patients using a previously developed ADA assay (21). This is much higher compared to three previously published certolizumab trials (2-4). Two of these studies did not report which assay had been used, making it difficult to compare results from one study to another. The high percentage of antibody positive patients in our cohort, could potentially be explained by the drug-tolerant assay we used (21).

Jani *et al.* used the same drug-tolerant one-step RIA for the detection of anti-certolizumab antibodies, as used in this study (4). The difference in the percentage (37% in the study by Jani *et al.* vs. 65% in our study) of patients in whom ADAs are detected might, partly, be due to differences in sampling strategy. In this study samples were taken at trough, as opposed to random sampling in Jani *et al.* Even with a drug-tolerant assay, drug levels will still influence detection of ADAs to certain degree. Furthermore, in the study by Jani *et al.* relatively more bDMARD naïve patients started certolizumab compared to our study (92% in the study by Jani *et al.* vs. 50% in our study). It might be that in our study some patients failed their previous bDMARD, due to immunogenicity. Therefore, these patients might be prone to develop ADAs against other bDMARDs (11, 30), further explaining the difference in the percentage of ADA positive patients.

The small sample size is a limitation of the present study. Consequently, a direct analysis of the relationships between certolizumab concentrations, anti-certolizumab antibodies and clinical response *i.e.* EULAR response or drug

adherence, could not be performed. Instead, we used TNF neutralising capacity as a functional outcome measurement. For a direct assessment of an association between certolizumab concentrations and clinical response, larger studies should be performed.

Of note, anti-certolizumab antibody titres were also determined using a two-tiered screening and confirmation approach, conform the FDA guidelines (31), but this appeared to have little consequences for the overall number of samples identified as positive (Supplementary Fig. S1).

In conclusion, we demonstrated that although a large proportion of patients has detectable anti-certolizumab antibodies high concentrations of certolizumab were found in most patients. We showed that drug concentrations, but not the presence of anti-certolizumab antibodies, was highly correlated with the capacity to neutralise TNF. So, serum drug concentrations reflect clinical effectiveness, which indicates that drug measurements can be used for the identification of under treatment. Overall, we advocate not to measure ADAs in a clinical setting, unless certolizumab concentrations are very low.

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